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13. ABSTRACT (Maximum 200 Words)

In this research project, we have established a yeast functional assay for the detection of p53 mutations from formalin fixed, paraffin imbedded, archival prostate cancer (CaP) tissue. We have demonstrated that the system is more sensitive than SSCP. We have utilized the system to evaluate the dominant negative gain-of-function effects of p53 mutations found in CaP tissue. We have shown that this method can be used for other tissues such as benign prostatic hypertrophy (BPH), lung cancer, and bladder cancer. This will allow investigators to carry out retrospective studies concerning p53 mutations utilizing paraffin imbedded rather than frozen tissue.

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Introduction

The subject of the research is *"Functional Detection of p53 Mutations in Archival Prostate Cancer Tissue"*. Studies have shown that *p53* mutation may be a useful marker in the progression of prostate cancer (CaP). The purpose of this research was to establish a yeast functional assay for detection of *p53* mutations from formalin-fixed, paraffin-embedded archival CaP tissues. The yeast functional assay developed by Dr. Iggo has been shown to be more sensitive than other methods in detection of *p53* mutation (1-3). However, it cannot be used for analysis of genomic DNA due to the inability to splice exogenous DNA into mRNA in yeast cells. By the support of the award, we have establish a yeast function assay and have been able to analyze the genomic *p53* DNA extracted from paraffin embedded tissues. Using this assay, we can detect the *p53* mutations from archival CaP tissues to test its efficiency and application. Microdissection was used to enrich for tumor cells, thus permitting us to find *p53* mutations in small tumor foci. Finally, we determined the functional status of several *p53* mutations found in CaP, including dominant-negative effects and gain-of-function activity. Because these functions of mutant *p53* gene are important in tumorigenesis and progression of cancer, these results help us to understand the biological function of individual *p53* mutations in CaP.

Body: Research and results

1. *To develop yeast function assay that is able to identify p53 mutations in paraffin-embedded prostate cancer tissues.*

We established a yeast assay that can be used to functionally detect *p53* mutations from formalin fixed, paraffin-embedded archival CaP tissues by modifying the Flaman's approach that has been used to analyze mRNA, not genomic DNA, extracted from frozen fresh tissues (4). The modification includes: *a*) microdissection of CaP tissues to enrich the tumor genomic DNA, *b*) PCR amplification of individual *p53* exons 5, 6, 7 and 8 from genomic DNA using *Pfu* DNA polymerase that has the proof-reading activity to diminish the PCR-induced errors, *c*) PCR-ligation of individual *p53* exons tested with the wild-type exons of *p53* gene to generate a large *p53* fragment that can be used for yeast transformation. Thus, the modified yeast assay is suitable for functional detection of *p53* mutation existing in genomic DNA. The development of the assay has been described in the previous report. The research accomplishments are as follows:

i) The primers required in the research have been designed and synthesized. The target *p53* exons 5, 6, 7 and 8 can be specifically amplified using corresponding primer pairs.
ii) PCR-mediated recombination of a large *p53* fragment encoding codons 53-364 has been established by using individual *p53* exons 5, 6, 7 or 8 and the wilt-type exons of *p53* gene.
iii) Yeast function assays of *p53* exons 5, 6, 7 and 8 amplified from the normal genomic DNA have been performed to determine the percentages (background levels) of white, red and pink yeast colonies. Based on these investigations, we accepted 2% or more pink colonies as indicating partial transactivational function and 12% or more red colonies as indicating loss of the function.
iv) Microscope-dissection, PCR amplification and the yeast function assay of *p53* have been established and optimized.

2. *To detect p53 mutations in archival CaP tissues using the modified yeast functional assay.*

Here, we addressed two issues: *a*) if the modified yeast assay could be used to screen for *p53* mutations from archival prostatic tissues, and *b*) if this assay was more efficient than the

single-stranded conformational polymorphism (SSCP) assay in identification of *p53* abnormalities. Thus, we first analyzed *p53* exon 5 and 6 in 11 archival prostate cancer tissues. From these tumors, four *p53* mutations were identified by the yeast assay, while three of the four mutations were found by SSCP assay. These results show that the yeast assay is more sensitive than the SSCP assay. The research accomplishments had been reported in the previous report.

Next, we examined four prostate cancer cell lines (LNCaP, PC3, TSU-Prl and DU145) to further determine the efficiency of the yeast assay. Cells were formalin-fixed, and paraffin-embedded. The genomic DNA was extracted and the yeast assay was performed. As anticipated, LNCaP cells have a silent *p53* mutation at codon 152 (CCG→CCA) of exon 5. Next, the *p53* exon 5 amplified from the LNCaP genomic DNA was examined using the modified yeast assay, 93% white colonies were yielded. This indicates that the silent mutation does not alter the transactivational function of expressed p53 protein. PC3 cells have a base deletion at codon 138 (GCC→GC) of *p53* gene, resulting in a frameshift, while TSU-Prl cells have a nonsense mutation at codon 126 (TAC→TAG). Analysis of the *p53* exon 5 from both cell lines produced 100% red yeast colonies, indicating total loss of transactivational activity. DU145 cells contain two missense mutations: CCT→CTT at codon 223 of exon 6 and GTT→TTT at codon 274 of exon 8. The two exons were separately amplified and examined in yeast. The mutation in exon 6 yielded 63% white, 11% red and 26% pink colonies, indicating that the resultant mutant protein retains partial transactivational activity. The mutation in exon 8 yielded 10% white and 90% red colonies, indicating a loss-of-function. These investigations further support the efficiency of the yeast assay.

We also examined 13 archival prostate tissues in which no *p53* abnormality was detected previously by both SSCP and immunohistochemistry. The genomic DNA was extracted from microdissected tumor cells and individual *p53* exons 5, 6, 7 and 8 were amplified by *Pfu* DNA polymerase-mediated PCR. After yeast transformation, none of the 13 tissues yielded more than 12% red colonies and more than 2% pink colonies. These results suggest that the modified yeast assay may yield, if any, very low level of false positive in screening for *p53* abnormality.

Table 1. Mutations detected from human prostate cancers: cell lines and archival tissues.

Sample	Exon/ mutation	Yeast assay			Mutation (allele, base change, aa change)	Transactivational Function
		White	Red	Pink		
LNCaP	5	93%	7%	0	P152P, CCG→CCA, Pro→Pro	normal
PC3	5	0	100%	0	L138, GCC→GC, frameshift	loss
TSU	5	0	100%	0	Y126, TAC→TAG, Tyr→stop	loss
DU145	6	63%	11%	26%	P223L, CCT→CTT, Pro→Leu	Partial
	8	10%	90%	0	V274F, GTT→TTT, Val→Phe	loss
1	5	85%	11%	4%	R158C, CGC→TGC, Arg→Cys	Partial
2	5	67%	33%	0	H179L, CAT→CTT, His→Leu	loss
3	5	82%	18%	0	P152S, CCG→TCG, Pro→Ser	loss
4	5	54%	45%	1%	H179R, CAT→CGT, His→Arg	loss

In addition, Dr. Gumerlock has used this assay to examine 5 archival BPH prostatic tissues for *p53* abnormality. The pure BPH cells were selected by laser-capture microdissection followed by genomic DNA extraction. The yeast assay of these BPH cells was performed and two mutations were identified (data not included).

Taking together, these investigations have shown that the modified yeast assay is efficient in identification of the *p53* abnormality. Several *p53* mutations have been identified from the archival prostate tissues and the embedded prostate cancer cells (**Table 1**). They have been merged into our CaP-derived *p53* mutation database for investigation of their function profile.

3. To determine dominant-negative effects and gain-of-function of p53 mutations found in CaP tissues.

From our *p53* mutation database, 16 CaP-derived mutant alleles were selected for determining their dominant-negative effects and gain-of-function activity. These alleles and their transactivational activity are listed in **Table 2**. Of them, 6 mutations were found in

Table 2. Prostate cancer-derived *p53* mutant alleles

Alleles	Base change	Original tumor	T.A. activity	mt spectrum
Y126C	TAC→TGC	Metastasis	Loss	
P151S	CCC→TCC	Metastasis	Loss	
R175H	CGC→CAC	Primary	Loss	hotspot
C176F	TGC→TTC	Metastasis	Loss	hotspot
C176S	TGC→TCC	Metastasis	Loss	hotspot
H214R	CAT→CGT	Primary	Partial	
P223L	CCT→CTT	DU145	Partial	
M237L	ATG→CTG	Primary	Partial	hotspot
G245S	GGC→AGC	Primary	Loss	hotspot
R248G	CGG→GGG	Metastasis	Loss	hotspot
R248W	CGG→TGG	Metastasis	Loss	hotspot
R273C	CGT→TGT	Primary	Loss	hotspot
R273H	CGT→CAT	Metastasis	Loss	hotspot
V274F	GTT→TTT	DU145	Loss	
R282Q	CGG→CAG	Metastasis	Loss	hotspot
Δ63-78	Δ16aa	primary	Partial	

primary CaP and 10 in metastatic CaP. In addition, 10of 16 alleles (R175H, C176F, C176S, M237L, G245S, R248W, R248G, R273H, R273C and R282Q) contain mutations in the "hotspot" sites of *p53* gene (**5**). Of the 16 mutant alleles, 12 were determined to have complete loss of transactivational activity, producing red yeast colonies while the remaining four were found to be able to induce the reporter gene to varying extents, yielding pink colonies and indicating partial function.

Dominant negative effect of p53 mutants

Mutant p53 can exert a dominant negative effect on wt p53. This phenomenon is thought to be mediated through the hetero-oligomerization between wild-type and mutant p53 protein. To determine the ability of p53 mutant to dominate over the wt p53 protein, the mutant *p53* alleles were analyzed in yeast using the approach described by Dr. Brachmann who kindly provided us all the plasmids and yeast strains (**6**). To test the dominant negative status, the diploid yeast cells tranformed with both mutant and wild-type *p53* expression plasmids were replica plated onto SC-His-**Leu**+ 0.15%Foa and SC-His-**Leu-Trp**+0.15%Foa. These plates were grown at 30°C for 3-5 days. Any growth on the SC-His-**Leu**+Foa plates indicates that mutant p53 is able to

dominant over 1 copy of wild-type p53 (class II). Any growth on the SC-His-**Leu-Trp**+Foa plates indicates that mutant p53 is able to dominate over 2 copies of wild-type p53 (class I). The experiment was repeated three times, and the same results were obtained. The results are illustrated in **Figure 1** and summarized in **Table 3**.
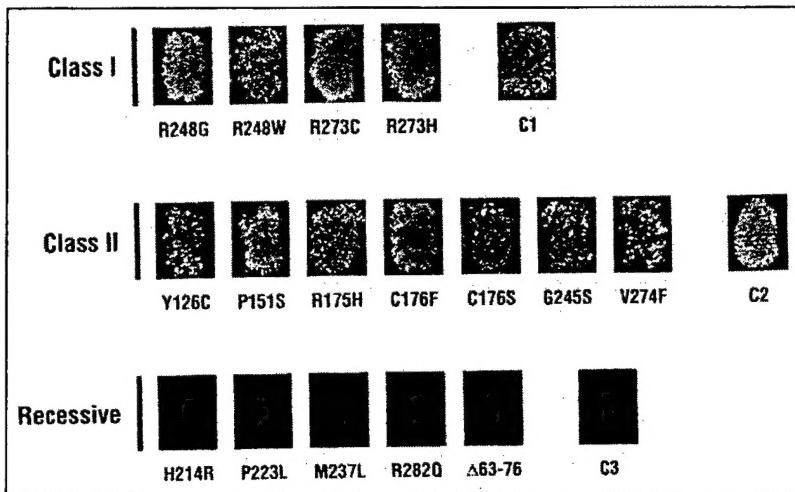

Figure 1. Dominant-negative assay of p53 mutants in yeast. *Top panel*. The yeast expressing the indicated p53 mutants were mated to yeast expressing two copies of wild-type p53. The resulting diploid yeast grew on SC-His-**Leu-Trp**+Foa selection medium, indicating that these mutants dominate over two copies of wilt-type p53 (class I). *Middle panel*. The yeast expressing the indicated p53 mutants were mated to yeast expressing one copy of wild-type p53. The resulting diploid yeast grew on SC-His-**Leu**+Foa selection medium, indicating that these mutants dominate over one copy of wilt-type p53 (class II). *Bottom panel.*The yeast expressing these p53 mutants failed to grow on either of the selective media and were considered to be recessive. C1, C2 and C3 are the corresponding controls

Four mutant *p53* alleles were found to be Class I dominant negative. The four mutants affect only two amino acid residues: 248 and 273 which are known to be in direct contact with the p53-responsive element (**7**). Thus, the protein products of the four Class I mutant alleles, when tetramerized with wt p53 protein, are not able to bind to target DNA, resulting in their stronger dominant negative phenotype. An additional seven mutants were found to be Class II dominant negative alleles that dominate over one copy of wt p53. These alleles contain the altered bases affecting the conformation of p53 protein. The remaining five alleles were found to be recessive to wt p53. Of these recessive alleles, four have partial transactivational function, suggesting that the partial-function p53 mutants do not dominate over wt p53. This may be because the resulting heterotetramer retains a almost normal conformation and the ability to bind target DNA. Since CaP growth is a fairly slow process and the frequency of loss of herozygosity (LOH) in chromosome 17p occurs only in 11% of CaP (**8**), our finding that certain p53 mutants exhibit the dominant-negative activity may be important in understanding the development of prostate tumor.

In vitro determination of gain-of-function activity of p53 mutants
(A) Ability of p53 mutants to upregulate MDR-1 and PCNA promoters.

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Since the promoters of the human multiple drug resistance gene 1 (MDR-1) and the human proliferating cell nuclear antigen (PCNA) genes can be activated by certain p53 mutants (**9, 10**), the two promoters were selected. To examine the gain-of-function activity of CaP-derived p53 mutants, several *p53* mutant allele with loss-of function were cloned into the pCR 3.1 vector (Invitrogen, Carlsbad, CA) under the control of a pCMV promoter. This plasmid was transiently co-transfected into Saos-2 cells with reporter plasmid carrying the MDR-1 or PCNA promoter fused to the CAT gene (kindly provided by Dr. Levine and by Dr. Mathews, respectively), as well as with a luciferase reporter plasmid that was used to normalize the transfection efficiency. The cells were grown for 36-40 hours at 37°C, 5% CO_2. A CAT-ELISA system (Roche Molecular Biochemicals, Indianapolis, IN) was used to analyze the activity of CAT enzyme produced as a result of the p53 mutant allele transactivating the MDR-1 or PCNA promoters. The entire experiment was repeated a minimum of 4 times for both MDR-1 and PCNA and the results averaged. Our results indicate that nine *p53* mutants were able to up-regulate the *MDR-1* promoter by at least 3-fold over wt p53, while four mutants were able to up-regulate the *PCNA* promoter greater than 2-fold over wt p53 (**Table 3**).

B) Potential of *p53* mutant-transfected cells to grow in soft agar.

Since untransfected *p53*-null Saos-2 cells do not grow in soft agar, colony formation caused by the presence of mutant p53 implies that the cells have gained a new function (**10**). We thus completed the soft agar assay in the 12 stably Saos-2 sublines. Soft agar plates were prepared

Table 3. Dominant-negative activity and gain-of-function of CaP-derived p53 mutants

Mutant Alleles	Transactivational activity	Dominate negative	Gain-of-function		
			MDR-1	PCNA	Soft agar
Y126C	Loss	Class II			+
P151S	Loss	Class II			
R175H	Loss	Class II	+		+
C176F	Loss	Class II	+		
C176S	Loss	Class II			
H214R	Partial	Repressive			+
P223L	Partial	Repressive	+	+	
M237L	Partial	Repressive		+	
G245S	Loss	Class II	+		+
R248G	Loss	Class I	+		
R248W	Loss	Class I	+		
R273C	Loss	Class I	+		+
R273H	Loss	Class I	+		
V274F	Loss	Class II			+
R282Q	Loss	Repressive	+	+	+
Δ63-78	Partial	Repressive		+	+

(using 60 mm² tissue culture dishes. Three ml of 0.6% Noble agar containing RPMI and 10% fetal bovine serum were poured into each plate to form a base. Cells of interest were grown in tissue culture as above and counted using a haemocytometer. 2×10^4 cells were diluted in 1 ml RPMI medium and then further diluted in 1 ml of the 0.6% Noble agar to give a final agar concentration of 0.3%. The cell mixture was poured on top of the hardened agar base and allowed to solidify. The plates were grown at 37°C in a 5% CO2 humidified atmosphere for

three weeks before colonies were counted. The ratio of cells able to form colonies in soft agar was calculated by dividing the total number of colonies formed by the number of cells plated. We observed that eight formed colonies in soft agar at frequencies greater than 1%. **Table 3** summaries the quantitative analysis of the gain-of-function activity for these *p53* mutants.

Key Research Accomplishments
- The specific primers amplifying the *p53* exon 5, 6, 7 or 8 have been synthesized.
- The methods of microdissection and different PCR conditions have been optimized.
- The yeast functional assays of genomic *p53* DNA have been developed.
- The analysis of the wild-type exons 5, 6, 7 and 8 of *p53* gene have been performed. The background levels of red and pink yeast colonies and the cut-off values for scoring loss-of-function and partial function mutations have been determined.
- Using this assay, 24 archival CaP tissues and four cell lines have been analyzed. Several *p53* mutations have been identified and collected in our p53 mutation database for further evaluating their functions.
- The dominant-negative effects of 16 CaP-derived p53 mutants have been evaluated in yeast. The positive results were obtained.
- The gain-of-function activity of the 16 mutants has successfully evaluated.
- One manuscript has been submitted recently and another is being prepared.

Reportable Outcomes
Abstracts
1. A new method of evaluating archival prostate cancer tissue for functionality mutant *p53* alleles. Di Mauro SM, Shi XB, deVere White RW, Evans CP. Presented at 5th Cancer Research Symposium. University of California Davis, Cancer Center, Sept 17-18, 1999, Sacramento.
2. The identification of functionally mutant p53 alleles in archival prostate cancer tissue. Di Mauro SM, Shi XB, deVere White RW, Evans CP. Presented at the Western Section AUA, Sept 28, 1999.
3. Loss-of-function, dominant-negative and gain-of-function mutant p53 alleles in human prostate cancers. Shi XB, Nesslinger N, Deitch AA, Gumerlock PH, deVere White RW. Proceeding of the American Association for cancer research, 41: 821, 2000.

Manuscripts
1. Application of the yeast assay to detect functional p53 mutations in archival prostate cancer tissue (in preparation)
2. Shi XB, Nesslinger N, Deitch AA, Gumerlock PH, deVere White RW. Complex functions of mutant p53 alleles from prostate cancer. (submitted to Oncogene)

Employment opportunity
Xu-Bao Shi, assistant research biologist, is engaged in the project and 60% of his salary comes from the award.

Research opportunity
Steve Di Mauro, Student of Medical School, was supported to conduct the research for one year.
Nancy Chen, undergraduate student, was trained in part time for one year under the award.
Will Holland, Lab technician, was trained to detect the p53 mutations from archival BPH tissues

Conclusions

1. Supported by this award, we have developed a yeast function assay that permit us to functionally detect *p53* mutations in genomic DNA extracted from archival CaP tissue, while the current yeast assay can not apply to genomic DNA.
2. We have completed the evaluation of the application of this method by examining archival CaP tissue, and mutations of *p53* were identified. We observed that the yeast assay is more sensitive than SSCP in detection of genomic *p53* mutations.
3. The technique can be applied to different archival tissues.
4. By functional evaluation of 16 *p53* mutant alleles, it was concluded that different p53 mutations lead to different dominant-negative and gain-of-function activity.

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Appendices

Personnel receiving pay from the research effort:
Dr. Xu-Bao Shi – Assistant Research Biologist (60% Effort)

Manuscript:
Complex Functions of Mutant p53 Alleles from Human Prostate Cancer.

Complex Functions of Mutant *p53* Alleles from Human Prostate Cancer

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Running title: Functions of mutant *p53* alleles in prostate cancer.

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ABSTRACT

Many studies have identified *p53* mutations in human carcinoma of the prostate (CaP), however few have addressed the functional status of these mutant alleles. In this study, we examined 16 CaP-derived mutant *p53* alleles for loss-of-function, dominant-negative and gain-of-function status. A yeast assay measuring their transactivational activity revealed that twelve mutant alleles resulted in total loss of this function, while 4 mutant alleles retained partial transactivational function. The partial function mutants were temperature sensitive: two were heat sensitive and two were cold sensitive. Of four temperature sensitive mutants, three were derived from localized CaP. Dominant negative status was determined for the 16 *p53* alleles using a second yeast assay, with 4 classified as Class I (dominating over two copies of wild-type p53), 7 as Class II (dominating one copy of wild-type p53) and 5 as recessive. Gains of function of these mutant *p53* alleles were measured in human cells. Utilizing *p53*-null mammalian Saos-2 and PC3 cell lines transfected with the *p53* mutant alleles, we demonstrated that gains of function are independent of loss of function. In Saos-2 cells transfected with the *MDR-1* or *PCNA* promoters, many of these alleles were able to up-regulate one or both of these promoters. In addition, 5 of 10 Saos-2 sub-lines showed increased expression of IL-6. All five alleles from localized CaP and 3 of 7 of those from metastatic CaP conferred the ability of Saos-2 cells to grow in soft agar. These mutations had the ability to induce *IL-6*, *MDR-1* or *PCNA* to varying degrees. They also demonstrated altered cell cycle profiles with increased S-phase fractions for 4 mutants derived from metastatic CaP. These results provide evidence that different *p53* mutations from CaPs have widely different, complex functional profiles. If we are to understand the role of mutated p53 in the initiation, progression and response to treatment of this disease, each individual *p53* mutation will have to be assessed for its functional alterations.

INTRODUCTION

The *p53* tumor suppressor gene is a transcription factor that functions in a network of multiple signaling pathways in response to DNA damage (Vogelstein *et al.*, 2000; Agarwal *et al.*, 1998; Somasundaram, 2000). The protein product of the *p53* gene has been shown to control cell cycle progression, DNA repair and cell survival (Levine *et al.* 1991; Lowe *et al.*, 1993). The most common genetic alteration thus far observed in human cancers is *p53* mutation which occurs in more than half of all tumors (Hussain and Harris, 1998). Approximately 90% of these are missense mutations in the evolutionarily-conserved, central DNA-binding core domain responsible for the sequence-specific binding of wild-type (wt) p53 protein to target genes (Cho *et al.*, 1994; Hussain and Harris, 1998). These mutations result in single amino acid changes that influence the conformation of the mutant protein or its sequence-specific binding, thereby altering or abrogating its ability to induce transcription of target genes (Cho *et al.*, 1994). One frequent result of these mutations is total ablation of the transactivational ability of mutant *p53*, referred to as a loss-of-function phenotype. More rarely, certain amino acid substitution mutants exhibit partial function. The loss of transactivational ability of mutant *p53* may be accompanied by other changes that influence its multiple activities. The presence of a mutant *p53* allele can alter the behavior of the remaining wt allele in a dominant-negative fashion. Further, some mutant p53 proteins gain the ability to induce inappropriate target genes (Chin *et al.*, 1992; Margulies and Sehgal, 1993; Chevillard *et al.*, 1997; Lanyi *et al.*, 1998) and they may also acquire oncogenic potential, resulting in blocks to apoptosis, increased tumorigenesis or the ability to grow in soft agar (Dittmer *et al.*, 1993; Alsner *et al.*, 2000; Sigal and Rotter, 2000).

Individual functional changes have been documented for several specific mutant *p53* alleles. However, because of the broad range of known functions of *p53*, functional evaluation of this gene will require studying multiple effects and examining specific mutations in specific cancers. Here we present an in-depth study of a selected panel of *p53* mutations in prostate cancer (CaP).

Mutations of *p53* are common in prostate cancer (reviewed by Heidenberg *et al.*, 1996). Estimates from the American Cancer Society indicate that 30% of CaPs have *p53* mutations (Harris, 1996). Our previous surveys have found the frequency of *p53* alterations to be approximately 39% in surgically resected primary CaPs (Chi *et al.*, 1994). While some investigators have found *p53* alterations to be rare in early stage primary CaP, many observers believe that this results from intra-tumoral heterogeneity (Mirchandani *et al.*, 1995; Roy-Burman *et al.*, 1997) and other technical considerations (Bonner *et al.*, 1997). There is general agreement that late stage CaP exhibits a higher frequency of *p53* mutations. We identified *p53* mutations in 71% of CaP metastases to bone (Meyers *et al.*, 1998), while other investigators have also noted high frequencies of *p53* mutations in androgen-independent and metastatic CaP (Navone *et al.*, 1993; Heidenberg *et al.*, 1995; Eastham *et al.*, 1995). The high rate of mutant *p53* found in advanced CaP has suggested to a number of observers that these mutations may be involved in the metastatic process and in the progression from androgen-dependence to androgen-independence. Approximately 250 *p53* mutations have been identified in CaP (www.iarc.fr/p53/index.html, updated 04/00) including those in several "hotspot" codons that are unique to CaP (deVere White, *et al.* 1999). Since information concerning the functionality of *p53* mutations in prostate cells is so limited, it is not known how these mutants affect the biology of CaP.

The selection of CaP for an in-depth study of *p53* functionality is appropriate for a number of reasons. This is the most common cancer of adult males (Greenlee *et al.*, 2000) and, as mentioned above, approximately one-third of the cases will have *p53* mutations. Prostate cancer is slow growing and the routine use of serum prostate-specific antigen (PSA) to screen for CaP has resulted in detection of 80-90% of these cancers while still localized. Treatment failure after radical surgery as judged by a PSA value higher than baseline also occurs relatively slowly. It has been estimated that while 32% of those treated surgically will show this biochemical relapse within 5 years, at that time only 5% will have overt metastases and an additional 2% will have local recurrences (Epstein, 1998). Thus, in this disease the clinician has a broad window of opportunity in which treatment of specific CaP patients could be individualized. Although many authors have suggested that CaPs with mutated *p53* may be more aggressive, in view of the possible functional diversity of different *p53* alleles, the resolution of this issue has considerable clinical importance.

In the present study, we selected a panel of 16 *p53* mutations found in CaP. Multiple assays were used to examine the function of each mutant allele. Two yeast assays were employed to evaluate the loss-of-function and dominant-negative status of these alleles. Human cell assays were used to determine whether inappropriate target genes were being activated, whether the alleles tested could contribute to growth in soft agar, and whether changes in cell cycle distributions occurred.

RESULTS

Demonstrating loss of p53 transactivational ability in yeast

A yeast functional assay originally described by Flaman *et al.* (1995) was used to determine whether mutant p53 proteins encoded by CaP-derived alleles could transactivate a *p53*-response element. In this assay, the color of the yeast colonies indicates the extent of p53 function. Wild-type (wt) function results in white yeast colonies, partial function in pink colonies and loss of function in red colonies. To assess the false-positive background of the yeast assay, we examined the *p53* transcripts from four normal human tissues, skin, muscle, breast and liver. The yeast assay was repeated three times to determine the percentages of the different phenotypes. For these normal control tissues, we found $90 \pm 0.8\%$ white colonies, 0.3% pink colonies and $9.7 \pm 0.5\%$ red colonies (data not shown). Based on these background levels, the levels of 2% or more pink colonies and 12% or more red colonies were set for determining partial function and loss of function, respectively.

We began the study by examining the function of known mutant *p53* alleles found in four human CaP cell lines (Chi, *et al.*, 1994, Voeller *et al.*, 1994). LNCaP cells are known to contain two *p53* alleles, one wt and one with a silent point mutation in codon 152 (CCG→CCA: proline). The *p53* mRNA transcripts from this cell line yielded >95% white yeast colonies indicating that, as expected, both alleles produce p53 with wt function (Figure 1A). PC3 and TSU-Pr1 cells each contain one transcribed *p53* allele whose mRNA can be analyzed in this yeast functional assay. The PC3 transcript has a single base deletion from codon 138 (GCC→GC), resulting in a reading frameshift while the TSU-Pr1 transcript has a nonsense point mutation at codon 126 (TAC→TAG: stop). The results of the yeast assay for both cell lines were the same: both

yielded red colonies and are thus loss-of-function alleles. DU145 cells are known to contain two different missense mutant alleles (223: CCT→CTT, Pro→Lys; 274: GTT→TTT, Val→Phe). Analysis of the *p53* transcripts from DU145 yielded 71% red, 16% pink, and 13% white colonies. The *p53* inserts in plasmids extracted from each of three colonies from each phenotype were sequenced. The red colonies contained the mutant V274F allele, pink colonies contained the P223L allele, and white colonies were wt at both codons. The percentages of colonies for each phenotype described above are consistent with the observation that DU145 contains two copies of the V274F and one of the P223L allele (Voeller, *et al.*, 1994). Additionally, the presence of wild-type inserts as a result of this assay is consistent with previously described DNA mismatch repair that results in intra-allelic recombination when more than one allele is present in the yeast system (Flaman *et al.*, 1995).

Prostate cancer-derived p53 mutants exhibit different transactivational activities in yeast

To investigate whether CaP-derived *p53* mutations exhibit different transactivational activities, 16 mutant *p53* alleles were selected for study (Table 1). Fifteen of these alleles have also been found in other types of cancer. Five alleles (R175H, H214R, M237L, G245S, R273C), while occurring in other cancer types, have so far been only documented in primary prostate cancer. An allele containing a 48 bp deletion from codons 63-78 (Δ63-78) has so far only been found in primary prostate cancer. The remaining 10 alleles (Y126C, P151S, C176F, C176S, P223L, R248G, R248W, R273H, V274F and R282Q) have been identified in metastatic prostate cancer (deVere White *et al.*, 1999; www.iarc.fr/p53/index.html). Ten of the mutant alleles are considered to occur in "hotspots" in prostate cancer since that particular allele has been

identified in at least 3 different prostate cancer specimens. Nine of the 16 mutant alleles were previously identified in our laboratory and their cDNAs were cloned to generate the corresponding yeast expression plasmid. The remaining alleles, identified by other groups, were prepared by site-directed mutagenesis as described in Methods. Of the 16 mutant alleles, 12 had complete loss of transactivational activity, producing red yeast colonies, while the remaining four were able to induce the reporter gene to varying extents, yielding pink colonies indicating partial function (Table 2). The pink yeast colonies were of different shades, ranging from white-pink to reddish-pink, in the following order: M237L>Δ63-78>P223L>H214R, with M237L being closest to white and H214R being closest to red. Since the same results were obtained multiple times, these differences in color are interpreted as reflecting the levels of retained transactivational function.

Some mutant *p53* alleles have previously been found to be temperature sensitive. We therefore examined the temperature sensitivity of all 16 mutant *p53* alleles with this assay (Figure 1B, Table 2), and found that four were indeed temperature sensitive. The P223L and the H214R *p53* mutant alleles were heat sensitive. While they gave white colonies at 25°C, indicating normal transactivational activity, at 35°C these alleles gave reddish-pink and pink colonies respectively, indicating partial function. Unexpectedly, the other two partial-function alleles were cold sensitive. The M237L allele gave white-pink colonies at 35°C, but at 25°C the colonies were a deeper shade of pink. Similarly, the Δ63-78 yielded light-pink colonies at 35°C but red colonies at 25°C. In agreement with our results, the H214R allele has been previously reported to be heat

sensitive whereas the R175H, R248G, R248W and R273H alleles were reported not to be temperature sensitive (Jia *et al.*, 1997).

Dominant-negative effect of p53 mutants

Mutant p53 has been shown to exert a dominant negative effect on wt p53. Two classes of dominant negative alleles have been described: Class I alleles are able to dominate over two copies of wt p53, while Class II alleles can dominate over a single copy of wt p53 (Brachmann *et al.*, 1996). To determine the ability of mutant p53 to dominate over the wt p53 protein, the 16 mutant alleles were analyzed in a yeast assay that defines the different degrees of dominant-negative ability. This assay uses a *p53*-responsive minimal promoter to drive the expression of *URA3*, which is required for yeast growth. Activation of *URA3* leads to survival on medium lacking uracil but prevents growth on plates containing 5-fluoroorotic acid (Foa) due to conversion of Foa into a toxic product. The experiments were repeated three times with the same results, illustrated in Figure 2 and summarized in Table 2. Four mutant *p53* alleles were found to be Class I dominant negative. An additional seven mutants were found to be Class II dominant negative alleles. The remaining five alleles were recessive to wt *p53*. Of the latter group, four have partial transactivational function, implying that partial-function *p53* mutant alleles do not dominate over wt *p53*. Our classification of the R248W allele as a Class I allele concurs with previously published results of Brachmann *et al.* (1996). However, these authors described the G245S allele as a Class I dominant negative allele and we found it to be only a Class II dominant negative allele.

In vitro human studies

Stably transfected sub-lines of Saos-2 human osteosarcoma cells were created for 15 of the 16

mutant *p53* alleles. The expression of p53 in the sub-lines was confirmed by Western blotting

(data not shown). We were unable to establish a stable Saos-2 line containing the M237L allele

or, as has been widely reported, the wt *p53* allele (Dittmer *et al.*, 1993, Ponchel and Milner,

1998).

Increased basal levels of $p21^{CDKNI}$ caused by specific mutant p53 alleles in Saos-2 cells

The basal level of the cyclin-dependent kinase inhibitor $p21^{CDKNI}$ is controlled primarily by wt

p53 (Tang *et al.*, 1998). We therefore tested the ability of *p53* mutants to increase the basal

levels of $p21^{CDKNI}$ in the *p53*-null Saos-2 cells. Stable Saos-2 cell sublines containing the

different *p53* alleles were examined for $p21^{CDKNI}$ protein levels by Western blotting, which was

performed twice with similar results. Four of the alleles (C176S, R248G, P223L, and H214R)

were able to increase levels of $p21^{CDKNI}$ above the level found in untransfected Saos-2 cells

(Figure 3). Two of these alleles (P223L and H214R) also had shown partial transactivational

function in yeast. However, the other two alleles (C176S and R248G) showed promoter-

dependent, or cell-type specific, activity. For these alleles, no transactivation was detected in

yeast (which has 3 copies of the RGC *p53* consensus-binding site upstream of the promoter)

whereas they were able to transactivate the $p21^{CDKNI}$ promoter in Saos-2 cells.

Gain-of-function determination in Saos-2 cells

Previously published studies have found that several mutant *p53* alleles, including R175H,

R248W, R273H and R281G, are able to exert gain-of-function effects *in vitro*. However, most

of the 16 mutant *p53* alleles have not previously been examined for such activities. We therefore

investigated gain-of-function activities in transfected Saos-2 cells in three ways: by looking for

up-regulation of the multiple drug resistance gene-1 (*MDR-1*) and proliferating cell nuclear

antigen (*PCNA*) promoters, by induction of IL-6 expression and by determining colony forming

ability in soft agar. (*a*) *Up-regulation of the MDR-1 and PCNA promoters*. Since the promoters

of *MDR-1* and *PCNA* genes can be activated by certain *p53* mutants (Chin *et al.*, 1992; Deb *et

al.*, 1992; Subler *et al.*, 1992; Dittmer *et al.*, 1993; Goldsmith *et al.*, 1995; Shivakumar *et al.*,

1995; Lanyi *et al.*, 1998), these two promoters were selected for study. Co-transfections of

Saos-2 cells were carried out with a promoter-chloramphenicol acetyl transferase (CAT) reporter

construct and an individual *p53* mutant expression plasmid (or, as a negative control, an empty

expression plasmid), and the CAT activity was measured by ELISA. The experiment was

repeated 5 times for each promoter and the results averaged. Nine of the *p53* mutants were able

to up-regulate the *MDR-1* promoter by at least 3-fold over wt *p53* (Table 3). Three of the *p53*

alleles that we found to up-regulate the *MDR-1* promoter, R175H, R248W and R273H, had also

been shown by Dittmer *et al.* (1993) to up-regulate this promoter. The *PCNA* promoter is

differentially activated by wt p53 depending on the concentration of *p53* plasmid DNA. Wild-

type p53 activates the promoter at low levels, but inhibits it at high concentrations (Shivakumar

et al., 1995). At the low concentration that was used in this study (0.75 μg), we found the *PCNA*

promoter to be activated by wt p53 as compared to the expression vector alone. Most of the *p53*

mutant alleles activated the promoter at levels below that of wt *p53*, indicating loss of function in

these alleles; however, four mutants were able to up-regulate the *PCNA* promoter more than 2-

fold over wt *p53* (Table 3). (*b*) *Induction of IL-6 expression*. The human *IL-6* promoter has been

shown to be activated by mutant *p53* and repressed by wt *p53* (Margulies and Sehgal, 1993). The downstream effect of mutant *p53* on IL-6 expression was examined by ELISA in 10 of the stable Saos-2 sub-lines established. The ELISA was run in duplicate on two 48-hour supernatants and the results averaged. Expression of IL-6 was seen in 5 of the 10 sub-lines, while no expression of IL-6 was detected in the untransfected Saos-2 control (Figure 4). (*c*) *Colony formation in soft agar.* Soft agar growth assays are often used in place of, and correlate well with, tumorigenicity in nude mice. Since untransfected Saos-2 cells do not grow in soft agar, colony formation caused by the presence of mutant *p53* implies that these cells have gained new functions. We performed the soft agar assay in duplicate on 12 of the stably transfected Saos-2 sub-lines, and found that 8 formed colonies in soft agar at frequencies greater than 1% (Table 3). Dittmer *et al.* (1993) had reported growth of the R175H allele in soft agar at 0.28%, and reported this as a positive result. The differences in percentages obtained in the two studies may reflect differences in plating efficiency or other culture conditions.

Gain-of-function p53 mutants increase S-phase fraction in CaP cells

Analysis of the S-phase fraction, a parameter reflecting cellular proliferation, was examined to evaluate this gain-of-function activity of mutant *p53*s in prostate cells. We were able to establish stable sub-lines in the CaP cell line PC3 for 11 of the *p53* mutants. These were then used to determine S-phase fractions by flow cytometry. Three replicate runs of each sub-line were averaged. PC3 cells containing an empty vector was used as the control and was found to have an average S-phase fraction of 33.3% ± 2.37%. Four sub-lines, those containing the C176F, P223L, R273H and R282Q alleles, showed an increase of more than 5% (>2 standard deviations) in their S-phase fraction over that for the vector-only PC3 control. Representative histograms of

the vector-only PC3 control and PC3 containing the P223L allele are shown in Figure 5. The two sublines with the highest S-phase fractions (P223L and R282Q) were also shown to up-regulate the *PCNA* promoter as described above (Table 3). In addition, one subline, containing the R248W allele, had a greater than 5% decrease in S-phase compared to the vector-only control.

Functional profiles of localized and metastatic prostate cancers

A summary of the functional profiles for the 16 mutant *p53* alleles is presented in Figure 6. It is notable that the profiles generated by the different assays are extraordinarily complex for alleles derived from both primary and metastatic cancer. All alleles tested showed partial or complete loss of function using the yeast functional assay. When analyzing the gain-of-function results, while the patterns are different for each allele, except for the P151S allele all showed at least one gain of function. Unexpectedly, gains of function including growth in soft agar were common for alleles from both primary and metastatic CaP. Furthermore, for most alleles, from both primary and metastatic disease, multiple gains of function were found. Paradoxically, one allele from metastatic CaP, P151S, a loss-of-function, Class II dominant-negative allele, failed to show any gains of function. When the alleles are separated based on their derivation from localized or metastatic CaP, relatively few functional differences emerged. Three of four alleles showing partial function were found in alleles derived from primary CaP. All four partial function alleles were recessive in the test of allelic dominance. Finally, all the alleles that demonstrated significant gains in S-phase fractions were derived from metastatic CaP.

DISCUSSION

Approximately 250 different *p53* mutations have been documented in prostate cancer. The studies reported here are an in-depth functional analysis of a selected panel of mutant *p53* alleles found in CaPs. The studies were undertaken to determine if mutant *p53* alleles found in CaP were functionally equivalent or showed diverse biological effects. Because we suspected that there would be differences in function that correlated with varied cancer biology, we selected alleles identified *only* in primary CaPs or those found in metastatic CaP, both in hotspot and non-hotspot locations (deVere White *et al.*, 1999). The rationale was that there may be subsets of *p53* mutations that contribute to the metastatic phenotype through alterations resulting in gains of biological function or, alternatively, that do not contribute to an increased metastatic potential. This latter subset would not be expected to be found in metastatic CaP unless abnormalities in other metastasis-associated genes occurred.

Analysis of their transactivational ability in yeast provides a simple, reproducible color assay documenting loss of transactivational ability in the absence of mammalian regulators of *p53*. Intermediate color hues between white and red provide information about the level of retained activity. The addition of temperature as a variable further enhances the amount of information that can be extracted. While three-quarters of the alleles tested exhibited total loss of function, the remaining 25% were identified as partial function alleles. The latter produced a range of colors that were influenced by the temperature of yeast growth. Temperature sensitivity may provide information about the flexibility of the p53 protein (Ponchel and Milner, 1998). Most temperature sensitive alleles that have been described are located in positions that maintain the structural integrity of the β-scaffold (Cho *et al.*, 1994; DiComo and Prives, 1998). Previous

studies have also linked partial transactivational function to temperature sensitivity (Flaman *et al.*, 1995; Ponchel and Milner, 1998). In this study we have confirmed this latter observation and have identified a new class of temperature sensitivity, cold sensitivity. The color changes we observed for the heat-sensitive mutants are probably related to protein conformation. Heat sensitive mutants cause G1-arrest in tumor cells and have been used *in vitro* to demonstrate the role of p53 in the response of cell lines to chemotherapy and radiation treatment (van Laar *et al.*, 1996; Pollock *et al.*, 1998). While we do not yet have an explanation for cold sensitivity, we anticipate that such mutants may be useful reagents in studies of the interactions of p53 with other proteins. It is notable that three of the four partial function alleles are not in hotspot locations. The observation that three of the four partial-function alleles are found in primary CaP and are recessive alleles suggests that these may be markers of less aggressive CaP.

Mutant p53 has been shown to exert a dominant-negative effect over wt p53. This phenomenon may be mediated through hetero-oligomerization between wt and mutant p53 protein. The extent of inactivation of wt p53 by dominant-negative mutants depends on several factors. These include the ratio of wt to mutant sub-units in the tetramers, the conformation of the mutant protein, the degree to which the mutant p53 sub-units within the hetero-tetramer are able to bind to DNA, and the DNA binding site (Chene, 1998). The degree of dominant negative activity depends not only on the ability of the mutant protein to associate with and drive the wt protein into a mutant conformation, but also the ability of the mutant tetramer to bind DNA. Our finding that certain *p53* mutants exhibit dominant-negative activity may have importance in understanding the development of prostate tumors, since the disease process is slow and the loss of heterozygosity (LOH) in chromosome 17p occurs only in 11% of CaP (Massenkeil *et al.*,

1994). In this dominant-negative assay, the growth of diploid yeast is based on selection media. The simplicity of this assay is in contrast to those employing mammalian cells where dominant negative status has been shown to be influenced by the phenotypes of the cells employed (Forrester et al., 1995). The four Class I mutants that were studied affect only two hotspot amino acid residues: 248 and 273. These are known to be in direct contact with the p53-response element (Cho et al., 1994). Thus, the protein products of these four Class I mutant alleles, when tetramerized with wt p53 protein, are not able to bind to target DNA, resulting in their stronger dominant negative phenotype.

Paradoxically, the same alleles exhibiting loss of function in the yeast assay were shown in this study to have diverse gains of function. Nine of the 16 alleles up-regulated the MDR-1 promoter. Four up-regulated the PCNA promoter over the wt level, while the remaining alleles repressed this promoter as compared to wt p53. Half of the stable mutant p53 lines we established had increased expression of IL-6. Furthermore, growth in soft agar, the surrogate marker for tumorigenicity, was common, even for mutant alleles derived from primary prostate cancer. Moreover, partial function alleles also demonstrated similar patterns of functional gains.

While the prognostic meaning of this finding remains controversial (Menon, 1997; Stattin et al., 1997; Coetzee et al., 1997), the suggestion has been made repeatedly that aggressive CaPs are characterized by increased proliferation. Increased proliferation has been noted for higher grade and stage prostate cancer and for tumor progression (Nemoto et al., 1990; Visacorpi et al., 1991; Eskelinen et al., 1991; Harper et al., 1992; Visakorpi, 1992). It has also been found

in recurrent, localized CaP (Bettencourt *et al.*, 1996; Connolly *et al.*, 1997; Sakr and Grignon, 1997), and in metastatic CaP (Berges *et al.*, 1995). A previous study noted that *p53* mutants interacted specifically with two cellular promoters (p42 and p38) during the S-phase of the cell cycle, suggesting a role for mutant p53 in promoting cell growth (Chen *et al.*, 1994). Our group has previously published that the more aggressive CaPs of African American patients are marked by a combination of increased expression of the anti-apoptotic gene *bcl2* and increased S-phase fractions (deVere White *et al.*, 1998). It is therefore of considerable interest that the four mutant *p53*s that significantly increased the S-phase fractions when transfected into PC3 cells were all derived from metastatic CaP. Indeed, the finding of increased S-phase fractions is the only gain of function that was unique to the *p53* alleles derived from metastatic CaP.

The p53 gains of function studied are mediated by changed expression of a diverse group of genes that are expected to alter the behavior of multiple interconnected gene networks. To the well-known complexity of pathologic change occurring in CaP, we now add further functional complexity at the molecular level. The extraordinary finding that each p53 gain of function profile is different suggests the possibility recently presented in a review by van Oijen and Slootwig (2000), "Apparently every *p53* mutation confers different biologic properties that seem to influence the prognostic significance of mutated *p53* in tumors". Whether this occurs in CaP has yet to be determined. We plan to study a larger group of mutant *p53* alleles from patients with prostate cancer who have known outcomes. We will first determine whether each component of the p53 functional profile described here contributes unique information and will then ascertain how the resulting finding correlates with clinical outcome. In this

way, we will be able to determine whether the information obtained provides prognostic information. More importantly, these types of studies have the potential to provide information that will be useful in planning therapeutic strategies for the individual patient based on the *p53* status of their primary tumor.

MATERIALS AND METHODS

Cell lines

CaP cell lines DU145, LNCaP, and PC3, the human osteosarcoma cell line Saos-2, as well as the non-small cell lung carcinoma cell lines Calu-1 and A549 used as controls in certain experiments were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The TSU-Prl cells were a generous gift from Dr. Edward Gelmann. These cells were maintained in ATCC-recommended media supplemented with 10% fetal bovine serum and antibiotics. Cultures were routinely given fresh medium twice per week.

Site-directed mutagenesis

For each mutant allele, a complementary primer pair [a reverse primer (pR) and a forward primer (pF), both containing the specific base change] were required to generate the mutant *p53* allele. Four additional primers were used for every mutant allele: p53-S: 5'-ATGGAGGAGCCGCAGTCAGAT; p53-R: TCAGTCTGAGTCAGGCCCTT; p3: 5'-ATTTGATGCTGTCCCCGGACGATATTGAAC and p4: 5'-ACCCTTTTTGGACTTCAGGTGGCTGGAGTG. To generate each mutant, two PCR fragments were separately amplified from the wild-type *p53* coding region using the primer pairs p53-S/pR and pF/p53-R and *Pfu* DNA polymerase. The PCR products were purified to eliminate any wild-type *p53* template. The purified PCR fragments were joined together using the internal primer pair p3/p4 by a PCR ligation approach. The resulting p53 fragment was sequenced to confirm the presence of the desired mutation and the absence of PCR-induced mutations.

Yeast functional assay

The yeast functional assay of PCR-amplified *p53* cDNA was performed following Flaman's

method (Flaman *et al.*, 1995). The *p53* alleles were either created by site-directed mutagenesis

or cloned directly from the cDNA prepared from the prostate specimens from which they were

derived. In order to detect any temperature-sensitive phenotype, yeast cells transformed with

different *p53* alleles were plated on two dishes. One was grown at 35^0C and the other at 25^0C,

for three days. Color differences of yeast colonies between the two plates indicated that the

mutant *p53* allele was temperature-sensitive.

For plasmid extraction from yeast, single yeast colonies were digested with zymolyase (ICN,

Costa Mesa, CA). The *p53* expression plasmids were isolated using a Wizard Miniprep kit

(Promega, Madison, WI) and transformed into *E. coli* strain HB101. The plasmids were then

recovered, purified and sequenced on an ABI Prizm 377XL sequencer (Perkin-Elmer Applied

Biosystems, Foster City, CA).

Dominant negative assay

The dominant negative assay was carried out following the protocol described by Brachmann *et*

al. (1996). All the plasmids and yeast strains described below were kindly provided by Dr.

Rainer Brachmann. Briefly, each mutant *p53* allele was sub-cloned into the yeast plasmid

pRB16 and then transfected into the yeast strain RBy33 (*genotype*: MATa 1cUAS53::URA3).

The transfected cells were grown for 2-3 days at 35°C on SC-His plates. Six colonies from each SC-His plate were chosen and patched onto a fresh SC-His plate. At the same time, RBy160 (*genotype*: MATα 1cUAS53::URA3; *plasmid marker*:pLS76/ADH-p53 LEU2 CEN) and RBy161 (*genotype*: MATα 1cUAS53::URA3; *plasmid marker*:pLS76/ADH-p53 LEU2 CEN and pRB17/ADH-p53 TRP1 CEN) colonies were streaked over the entire surface of a YPD plate. These plates were grown at 35°C for 2-3 days. The RBy33 colonies from the SC-His plates were mated to both RBy160 and RBy161 on a fresh YPD plate. These mating plates were grown at 35°C for 2-3 days and then replica plated onto the following selective plates: SC-His-Leu for the RBy160-mated yeast and SC-His-Leu-Trp for the RBy161-mated yeast. The plates were grown at 30°C for 3-5 days, resulting in diploid cell growth only. To test dominant negative status, the diploid cells were replica plated onto SC-His-Leu+ 0.15%Foa (for the RBy160-mated yeast) and SC-His-Leu-Trp+0.15%Foa (for the RBy161-mated yeast). These plates were grown at 30°C for 3-5 days. Any growth on the SC-His-Leu+Foa plates indicate a Class II dominant negative status, while any growth on the SC-His-Leu-Trp+Foa plates indicates Class I dominant negative status.

Stable transfections of mutant p53 alleles into Saos-2 and PC3 cells

Each *p53* mutant allele was cloned into the pCR 3.1 vector (Invitrogen, Carlsbad, CA) that contains the pCMV promoter and was sequenced to ensure the presence of the mutation of interest. Each of the 16 mutant alleles was stably transfected into Saos-2 cells using 9 μg plasmid DNA and standard calcium phosphate precipitation with a glycerol shock at 5 hours post-transfection. Each of the 16 mutant alleles was also transfected into PC3 cells using

Lipofectin® Reagent (Life Technologies Inc., Gaithersburg, MD) and 4 µg plasmid DNA in serum-free media. Cells were grown for 5 hours at 37°C, 5%CO$_2$ before replacing the media with standard media containing 10% FBS. After 48 hours, both the transfected Saos-2 and PC3 cells were grown under G418 (500 µg/ml) selection for 2-3 weeks until isolated colonies appeared. Colonies were selected and expanded in 24 well plates before being transferred to culture flasks. Individual sub-lines were tested for the presence of p53 by Western blotting.

Western blotting

Whole cell extracts were prepared by lysing pelleted cells in cell lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% TritonX-100, 10 mg/ml leupeptin, 0.1M aprotinin, 0.1M PMSF, 0.1M NaVO$_4$, Rnase-free DNase). The tubes were left on ice for 30 min, and vortexed every 2-3 min. The cell lysates were spun at 12,000 rpm at 4°C and the cleared supernatants transferred to fresh tubes and stored at –70°C. Protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). 50-75 µg protein was separated on a 12% (for p53), or a 15% (for p21) SDS-PAGE mini-gel and transferred to Trans-Blot® nitrocellulose membranes (Bio-Rad, Hercules, CA). The p53 antibody (Ab-6, Calbiochem, San Diego, CA) was used at a 1:10000 dilution, the p21 antibody (PharMingen, San Diego, CA) was used at a 1:1250 dilution, and the β-actin antibody (Sigma, St. Louis, MO) was used at a 1:10000 dilution. Densitometric analysis of the p21 Western blots was performed using a BioRad GelDoc 1000 Documentation System (BioRad, Hercules, CA).

MDR-1 and PCNA CAT assays

Saos-2 cells were transiently co-transfected with the mutant *p53* expression plasmids and either the pMDR-1-CAT (provided by Dr. Arnold Levine) or pPCNA-CAT (provided by Dr. Jean Nussbaum and Dr. Michael Mathews) plasmids. Luciferase reporter plasmids were used to normalize the efficiency of transfection. A total of 2.5 µg plasmid DNA was transfected using 6 µl FuGene6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). The cells were grown for 36-40 hours at 37°C, 5% CO_2. A CAT-ELISA system (Roche Molecular Biochemicals, Indianapolis, IN) was used to analyze the amount of CAT enzyme produced as a result of the *p53* mutant allele transactivating the *MDR-1* or *PCNA* promoters. The entire experiment was repeated a minimum of 5 times for both MDR-1 and PCNA and the results averaged.

IL-6 ELISA

The IL-6 ELISA was carried out using 1×10^5 cells grown in 24 well plates in 500 µl standard RPMI medium. The cells were grown for 48 hours at 37°C, in a 5% CO_2 humidified atmosphere before harvesting the supernatant. An h-Interleukin-6 ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) was used to measure IL-6 protein in 20 µl conditioned media as per the manufacturers' directions. The experiment was carried out twice and the results averaged.

Soft agar assay

Soft agar plates were prepared using 60 mm^2 tissue culture dishes. Three ml of 0.6% Noble agar containing RPMI medium and 10% fetal bovine serum were poured into each plate to form a

base. 2×10^4 cells were diluted in 1 ml RPMI medium and then further diluted in 1 ml of the 0.6% Noble agar to give a final agar concentration of 0.3%. The cell mixture was poured on top of the hardened agar base and allowed to solidify. The cells on the plates were grown at 37°C in a 5% CO_2 humidified atmosphere for 14-21 days before colonies were counted. The ratio of cells able to form colonies in soft agar was calculated by dividing the total number of colonies formed by the number of cells plated.

Flow cytometry

Cells to be analyzed by flow cytometry were trypsinized, washed once in PBS and counted using a haemocytometer. Approximately 1×10^6 cells were placed in a polystyrene tube and washed once more with PBS. The cell pellet was resuspended in 2 ml cold 70% EtOH and kept at −20°C for at least one hour and up to 2 weeks. The cells were pelleted by centrifugation and washed once in PBS before resuspending in 880 µl PBS and 20 µl 0.5 mg/ml DNase-free RNase (Roche Molecular Biochemicals, Indianapolis, IN). The tubes were left at 37°C for 30 min before adding 100 µl 0.5 mg/ml propidium iodide (Roche Molecular Biochemicals, Indianapolis, IN). Cell cycle profiles were determined by standard flow cytometry using a Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL) and the Phoenix MultiCycle program (Phoenix Flow Systems, San Diego, CA) to determine the percentages of cells in the different cell cycle compartments.

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Table 1- Prostate cancer p53 mutations used in this study

p53 allele[1]	Mutation	Mutation found in:[2]
Y126C	TAC⇨TGC	metastatic
P151S	CCC⇨TCC	metastatic
R175H	CGC⇨CAC	primary (hotspot)
C176F	TGC⇨TTC	metastatic (hotspot)
C176S	TGC⇨TCC	metastatic (hotspot)
H214R	CAT⇨CGT	primary
P223L	CCT⇨CTT	metastatic (DU145 allele)
M237L	ATG⇨CTG	primary (hotspot)
G245S	GGC⇨AGC	primary (hotspot)
R248G	CGG⇨GGG	metastatic (hotspot)
R248W	CGG⇨TGG	metastatic (hotspot)
R273C	CGT⇨TGT	primary (hotspot)
R273H	CGT⇨CAT	metastatic (hotspot)
V274F	GTT⇨TTT	metastatic (DU145 allele)
R282Q	CGG⇨CAG	metastatic (hotspot)
Δ63-78[3]	Δ16αα	primary

[1] Mutations described in deVere White *et al.*, 1999.

[2] All mutations have been identified in either primary or metastatic prostate cancer and are considered hotspots if that amino acid is affected in more than 3 samples.

[3] Mutation previously unpublished.

Table 2- Loss of function and dominant negative results for 16 prostate cancer-derived p53 mutations as measured in yeast.

p53 Allele	Yeast Functional Assay	Temperature Sensitive	Dominant Negative
Y126C	LOF[1]	-	Class II
P151S	LOF	-	Class II
R175H	LOF	-	Class II
C176F	LOF	-	Class II
C176S	LOF	-	Class II
H214R	PF	heat sensitive	recessive
P223L	PF	heat sensitive	recessive
M237L	PF	cold sensitive	recessive
G245S	LOF	-	Class II
R248G	LOF	-	Class I
R248W	LOF	-	Class I
R273C	LOF	-	Class I
R273H	LOF	-	Class I
V274F	LOF	-	Class II
R282Q	LOF	-	recessive
Δ63-78	PF	cold sensitive	recessive

[1] LOF= loss of function, PF= partial function

Table 3- *In vitro* gain of function activity of *p53* mutant alleles as measured in Saos-2 cells

p53 Allele	MDR-1.CAT[1]	PCNA.CAT[2]	Soft Agar[3]
WT p53	1.0	6.1	nd
Y126C	2.8	4.9	6.70%
P151S	2.2	2.3	0.07%
R175H	3.0	2.1	1.91%
C176F	3.6	2.8	nd
C176S	2.8	2.8	0.79%
H214R	2.2	2.9	2.94%
P223L	3.1	15.3	0.45%
M237L	1.9	18.5	nd
G245S	13.0	3.7	1.64%
R248G	4.5	4.7	0.37%
R248W	6.0	3.2	nd
R273C	9.9	3.5	3.11%
R273H	3.8	3.2	nd
V274F	2.5	2.3	8.72%
R282Q	5.1	20.8	1.53%
Δ63-78	2.8	18.7	4.68%

[1] Represents the mutant p53-mediated increase in CAT activity compared to wt, averaged over 5 trials.

[2] Represents the p53-mediated increase in CAT activity compared to that of the expression vector alone, averaged over 5 trials.

[3] 2×10^4 cells were plated in soft agar and the percentage of cells that formed colonies of greater than 30 cells is presented.

FIGURE LEGENDS

Figure 1. A. Yeast assays for *p53* function. Analysis of mutant *p53* alleles in four CaP cell lines. LNCaP cells, containing 2 alleles encoding wild-type p53 protein, yielded 95% white colonies. Both TSU-Pr1 cells (*p53* nonsense mutation) and PC3 cells (frameshift mutation) resulted in 100% red yeast colonies. DU145 cells, with two missense mutations, gave white, red and pink colonies. **B.** Temperature-sensitive functional alterations of CaP-derived mutant *p53* alleles. The yeast transformants containing different mutant *p53* alleles were grown at 35°C and at 25°C. The control P152P allele with a silent mutation shows normal function at both the higher and lower temperatures. The Y126C and V274F alleles show total loss of function at both temperatures, while M237L, H214R, P223L and Δ63-78 show temperature-sensitive properties. Two types of temperature sensitivity were observed: H214R and P223L showed increased transactivational activity at the lower temperature (heat sensitive), while M237L and Δ63-78 showed decreased activity at the lower temperature (cold sensitive).

Figure 2. *p53* dominant-negative assay in yeast. *Top row:* The yeast strain RBy33 containing the indicated *p53* mutant alleles were mated to yeast containing 2 copies of wild-type p53. The resulting diploid yeast grew on SC-His-Leu-Trp+Foa selection plates, indicating that these mutants can dominate over 2 copies of wt p53 (Class I). *Middle row:* The yeast strain RBy33 containing the indicated *p53* mutant alleles were mated to yeast containing one copy of wt p53. The resulting diploid yeast grew on SC-His-Leu+Foa selection plates, indicating that these

mutants can dominate over 1 copy of wt p53 (Class II). *Bottom row*: These alleles were unable to grow on either of the selective media plates and are thus considered to be recessive. C1 is a known Class I dominant negative control, C2 is a known Class II dominant-negative allele and C3 contains a single copy of wt *p53* that is Foa-sensitive and thus does not form colonies.

Figure 3. Increased p21 basal levels caused by specific mutant alleles. Western blotting demonstrated the ability of specific mutant *p53* alleles to increase basal levels of p21. β-actin was used as the control to verify equivalent loading of proteins. A549 cells treated with cisplatin were used as the positive control while untreated Calu-1 cells were used as the negative control.

Figure 4. IL-6 expression is up-regulated by some mutant *p53* alleles. Levels of secreted IL-6 measured by ELISA are shown graphically. Five mutant alleles showed appreciable up-regulation of IL-6 in stably-transfected Saos-2 cells.

Figure 5. Increased proliferation of prostate cancer cells induced by specific mutant *p53* alleles. A representative cell cycle histogram of PC3 cells containing the P223L allele is compared to that for the PC3 vector-only control. Multicycle analysis revealed a significantly increased S-phase fraction for this, and for three other mutant *p53* alleles.

Figure 6. Functional differences between *p53* alleles derived from primary and metastatic CaP. The diverse functional profiles of the mutant alleles are summarized here. The gray rectangles indicate loss of function in the yeast assay. The diagonally-striped rectangles represent heat-

sensitive, partial-function alleles, while the vertically-striped rectangles represent cold-sensitive, partial function alleles. I = Class I dominant-negative alleles, II = Class II dominant-negative alleles, Rec = recessive alleles. The black rectangles represent positive results in the human cell assays, while nd = not done. No symbol indicates a negative result.

FIGURE 1
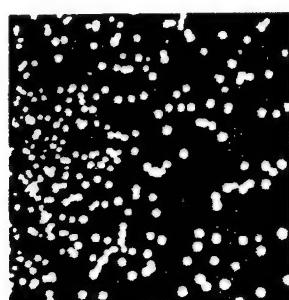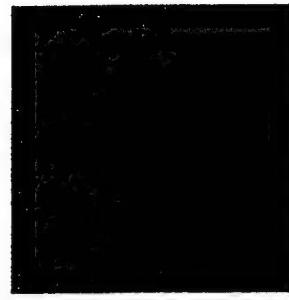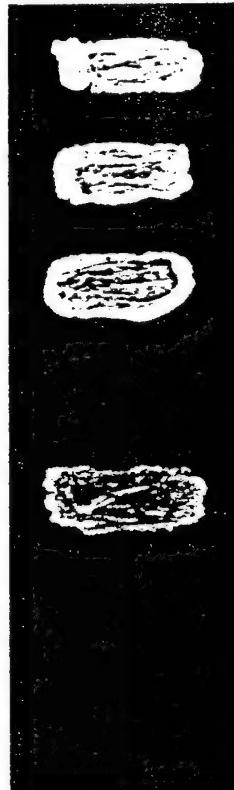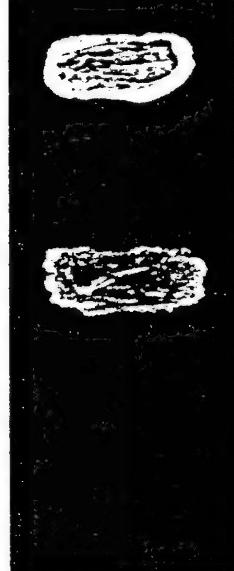
FIGURE 2

Class I

R248G   R248W   R273C   R273H   C1

Class II

Y126C   P151S   R175H   C176F   C176S   G245S   V274F   C2

Recessive

H214R   P223L   M237L   R282Q   Δ63-76   C3
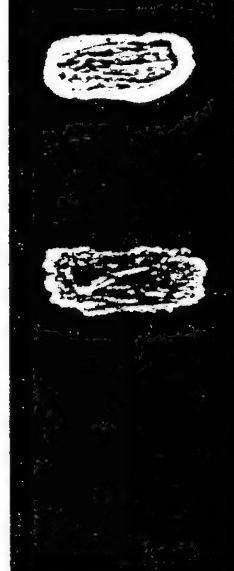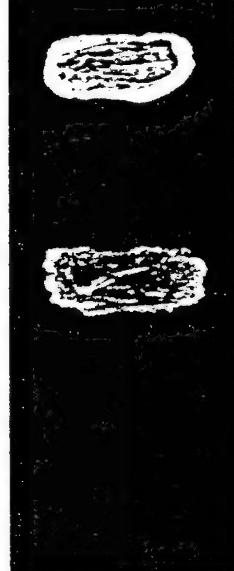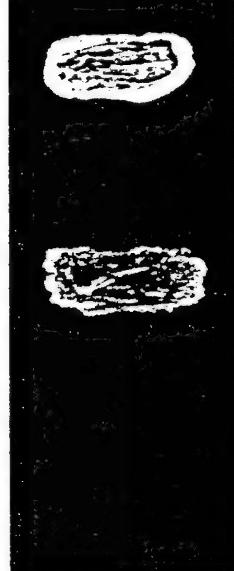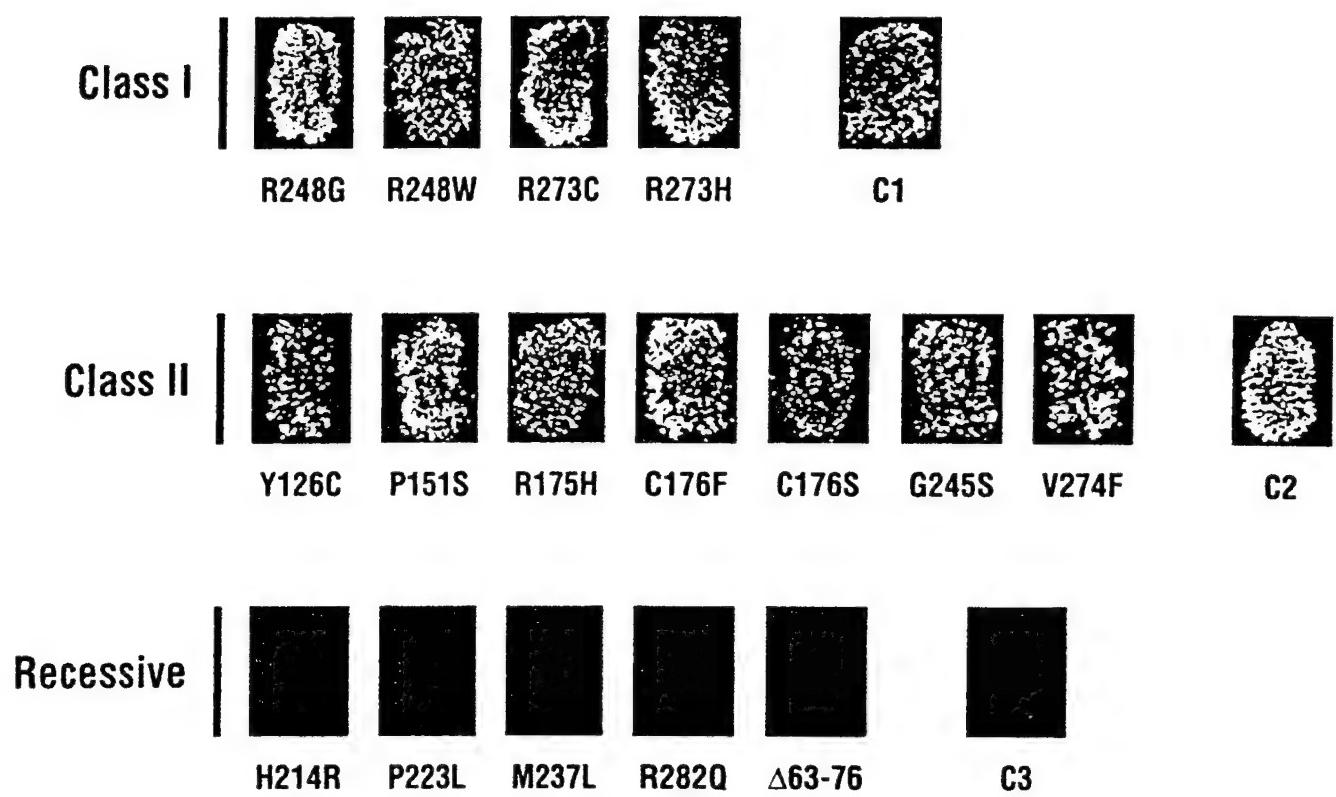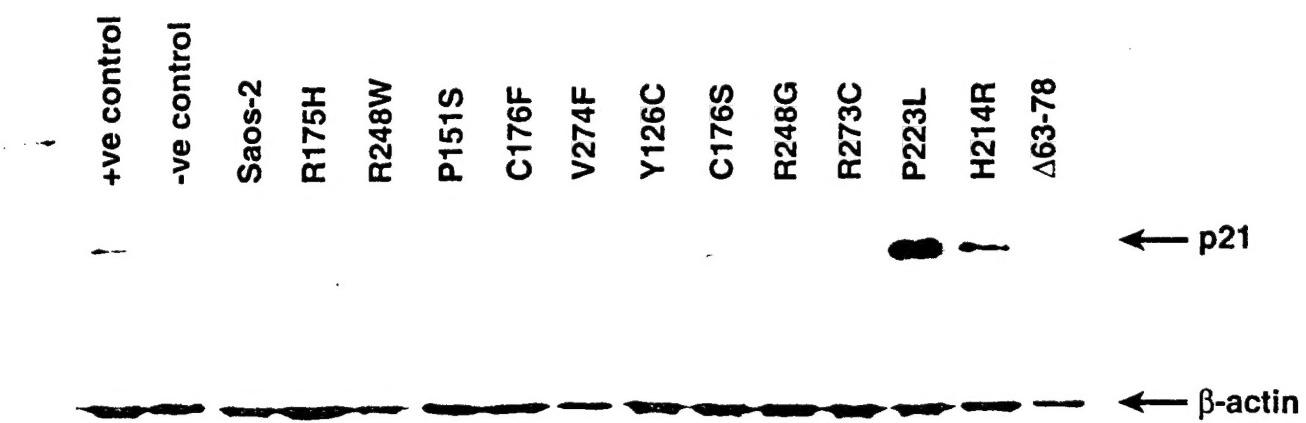
FIGURE 3

FIGURE 4

FIGURE 5

PC3 + P223L

376

PC3 control

611

FIGURE 6

	Yeast Cell Assays		Human Cell Assays				
p53 allele	LOF/PF	D/N	MDR-1	PCNA	IL-6	Soft Agar	S-phase
Primary prostate cancer							
R175H		II					
H214R		Rec			nd		
M237L		Rec			nd	nd	nd
G245S		II					
R273C		I					
Δ63-78		Rec			nd		nd
Metastatic prostate cancer							
Y126C		II					
P151S		II					
C176F		II			nd	nd	
C176S		II					nd
P223L		Rec					
R248G		I					
R248W		I			nd	nd	
R273H		I			nd	nd	nd
V274F		II					
R282Q		Rec					